Supplementary information for:

Collagen VI protects neurons against Aβ toxicity

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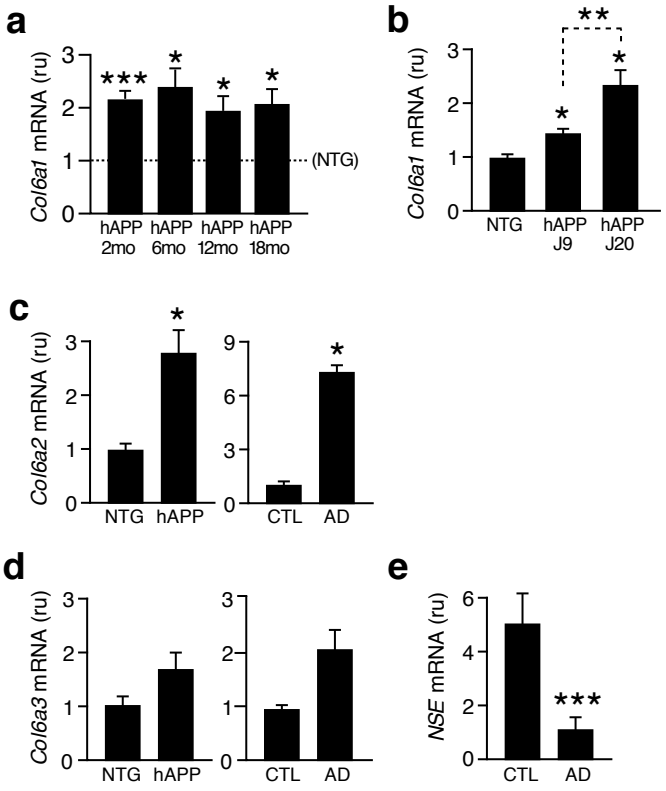
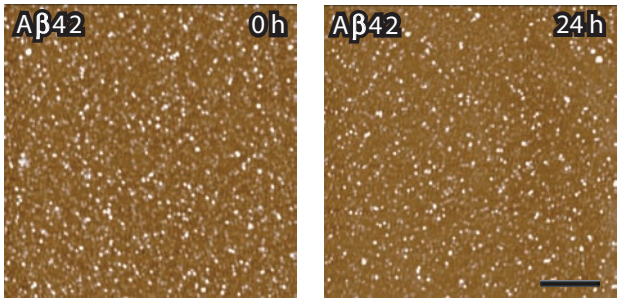
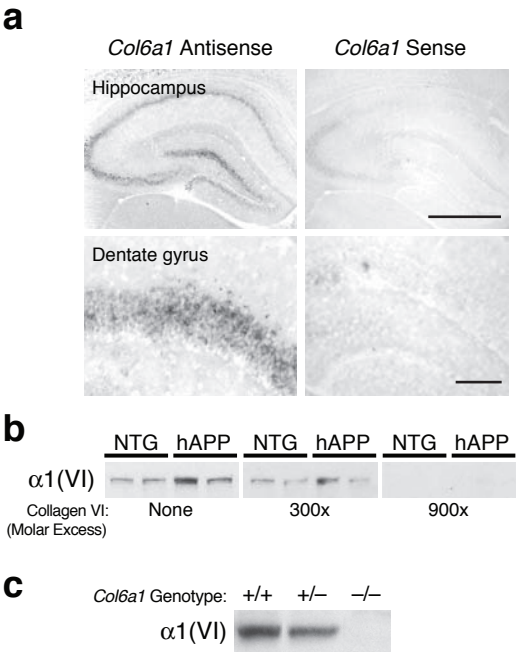


Supplemental Figure 1. Levels of mRNAs encoding collagen VI subunits were measured in the hippocampus or dentate gyrus of mice and humans by quantitative RT-PCR. (**a**) Hippocampal *Col6a1* mRNA levels in hAPP-J20 mice of the indicated ages (mo, months) relative to those in age-matched NTG mice (n=10-12 mice per group). *Col6a1* mRNA levels in NTG mice did not differ with age (data not shown). (**b**) Hippocampal *Col6a1* mRNA levels in 6-8-month-old NTG, hAPP-J9, and hAPP-J20A mice (n=8-10 per group). *Col6a2* (**c**) and *Col6a3* (**d**) mRNA levels in dentate gyrus of 3–6-month-old NTG and hAPP-J20 mice (n=11-13/group) and in dentate gyrus of humans with (n=8) or without (CTL, n=3) AD. *Col6 mRNA* levels, expressed as relative units (ru), were normalized to *GAPDH* mRNA levels in mice and to *neuron-specific enolase (NSE)* mRNA levels in humans, as described[1]. A neuronal house-keeping gene was chosen for normalization in human cases because collagen VI is likely produced primarily by neurons and AD is associated with significant neuronal loss. (**e**) *NSE* mRNA levels in the dentate gyrus of the human cases analyzed in (**c**) and (**d**). *P<0.05, **P<0.01, ***P<0.001 vs control (NTG or CTL) (t-test or Tukey test) or as indicated by bracket (Tukey test).



Supplemental Figure 2. Aβ42 aggregates were visualized by AFM. Aβ42 aggregates were deposited on mica after 0 h or 24 h of incubation *ex situ* (Supplemental Methods). At both time points, Aβ42 existed as small globular aggregates, confirming the oligomeric state of the Aβ42 preparations used in culture. Scale bar, 500 nm.



Supplemental Figure 3. Specificity assessment of the *Col6a1* antisense riboprobe and the α1(VI) antibody used in this study. (**a**) Coronal sections of the hippocampus and dentate gyrus from a hAPP-J20 mouse were analyzed by *in situ* hybridization with a *Col6a1*-specific antisense riboprobe (mCol6a1-4)[2] or with a complementary sense probe as indicated. (**b**) Preincubation (1 h) of the polyclonal anti-α1(VI) antibody with purified collagen VI eliminated immunostaining of α1(VI) bands on western blots of hippocampal homogenates from NTG and hAPP-J20 mice. (**c**) Reduction or ablation of the antigen resulted in a corresponding reduction or elimination of bands labeled with the anti-α1(VI) antibody on western blots of hippocampal lysates from mice with two (+/+), one (+/–), or no (–/–) functional *Col6a1* alleles. Scale bar, 500 μm.

SUPPLEMENTAL METHODS

hAPP Mice. We used 2–18-month-old male and female hAPP transgenic (TG) mice and nontransgenic (NTG) littermates from lines J20 and J9 on a C57BL/6J background[1-7]. TG and NTG groups were balanced for sex. Mice were anesthetized and perfused transcardially with phosphate buffer followed by 4% phosphate-buffered paraformaldehyde (PFA) (for *in situ* hybridization) or with phosphate buffer alone (all other studies). Hemibrains were fixed in 4% phosphate-buffered PFA (for histology) or stored at –80°C (for biochemical assays). All experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Human AD Tissue. Frozen blocks of human *postmortem* tissue containing dentate gyrus from AD cases and nondemented controls were from the New York Brain Bank at Columbia University Medical Center. Three nondemented men (age: 87.7 ± 1.1 years; CDR score: 0) and 8 AD patients (5 males, 3 females; age: 76 ± 3.6 years; CDR score: 3.0 ± 0.5) were included in this study. Brain pH, postmortem intervals, and RNA quality did not differ between the groups (data not shown).

Primary Cell Cultures. Combined cortices and hippocampi were isolated between embryonic day 18 and postnatal day 0 and used to establish mixed neuronal/glial cultures or cultures enriched for neurons (>95% immunoreactive for microtubule-associated protein-2 (MAP-2)) or astrocytes (>95% immunoreactive for glial fibrillary acidic protein) as described[8] from NTG wildtype, α1(VI)-deficient[9, 10], APP-deficient[11], and conditional TβRII-deficient mice lacking TβRII in forebrain neurons; the latter mice were generated by breeding mice carrying floxed TβRII alleles[12] with CamK-Cre93 TG mice expressing Cre recombinase directed by the calcium/calmodulin-dependent protein kinase II (CaMKII) promoter[13]. Mice were on the C57BL/6J background, except α1(VI)-deficient mice, which were on a mixed FVB/N and C57BL/6 background (crossed for 5 generations onto the C57BL/6J strain). Unless indicated otherwise, all treatments were carried out on day 6-7 *in vitro* in Neurobasal A medium supplemented with N2 (NBA/N2). All tissue culture reagents were from Invitrogen.

Culture Treatments and Cell Death Assay. Synthetic Aβ1-42 and reverse Aβ42-1 peptides (Biopeptide, San Diego, CA) were lyophilized in hydroxyfluroisopropanol (HFIP), reconstituted in dry dimethyl sulfoxide (DMSO) at 2.2 μM, diluted in NBA medium (pH 7.4) (Invitrogen, Carlsbad, CA) to 0.5 mg/ml, incubated at 4°C for 48 h, and stored at –80°C until use[14]. For treatment of cells, stock solutions of Aβ peptides were diluted in fresh NBA/N2 medium to final concentrations of 90 μg/ml or 45 μg/ml (equivalent in total Aβ content to 20 μM or 10 μM solution of monomeric Aβ). This type of preparation contained Aβ oligomers but no fibrils (**Supplemental Fig. 2** and ref.[5]).

Relatively high concentrations of Aβ were used here to achieve higher levels of injury over shorter periods of time than are likely to occur in the human condition in order to enable mechanistic studies and repeated experiments within a realistic time frame. It should be noted in this context that the concentration of Aβ oligomers in the local microenviroment of vulnerable neurons and synapses in the AD brain is unknown and could be much higher than concentrations measured in whole tissue homogenates. Furthermore, higher concentrations of synthetic than of naturally produced Aβ oligomers are required to achieve similar biological effects[15].

In toxicity studies, collagen VI (1-100 μg/ml, equivalent in content to 0-208 nM solution of monomeric collagen VI), collagen I (20 μg/ml, equivalent in content to 73 nM solution of monomeric collagen I), or vehicle (4 mM borate buffer for collagen pH 7-8 or 0.02 M acetic acid for collagen pH 2-3) dissolved in NBA/N2 medium was added to primary neuronal cultures from wildtype mice 1 h before exposure to oligomeric Aβ42 (45-90 μg/ml) or DMSO (final concentration 0.2%) without Aβ peptides. Purified human collagen VI (>95% pure), which has >86% sequence identity with mouse collagen VI[16], and purified human collagen I (both from Fitzgerald Industries, Concord, MA) were dissolved separately in N2 medium to final

concentrations of 1-100 μg/ml. SIS3 (EMD Biosciences, San Diego, CA), a specific inhibitor of Smad 3 phosphorylation[17, 18], was dissolved in DMSO and diluted in N2 medium (final concentrations: SIS3, 6 μM; DMSO, 0.02%). Lyophilized recombinant human TGFβ-1 (R&D Systems, Minneapolis, MN) was dissolved to 1 μg/ml in vehicle (4 mM HCl with 1 mg/ml bovine serum albumin) and then dissolved in N2 medium to a final concentration of 10 nM. After 24 h of neuronal exposure to Aβ peptides, cell death was measured with a trypan blue assay (Sigma-Aldrich, St. Louis, MO)[19]. In replicate cultures, cells and medium were harvested for RNA and protein analysis, respectively.

TGF-β Bioassay. TGF-β bioactivity in hippocampal extracts from NTG and hAPP-J20 mice (age 6-7 months), was assayed as described[20]. Briefly, hippocampi were dissected and homogenized in buffer containing 137mM NaCl, 20 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 10% glycerol and proteinase inhibitors (Roche, Indianapolis, IN). Samples were spun for 20 minutes at 2000 x g, and supernatant was acid activated with 2 μl of 6 M HCl for 10 min and then neutralized to pH 7.4 with 1.8-3.0 μl of 6 M NaOH. Samples were incubated for 24 h with MFB-F11 cells[20], embryonic fibroblasts from TGFβ1$^{-/-}$ mice stably transfected with a synthetic Smad-binding element promoter fused to a secreted alkaline phosphatase (SEAP) reporter gene. SEAP activity was measured with the Great EscAPe SEAP Reporter System 3 (BD Biosciences, San Jose, CA) and quantified with an Lmax plate photometer (Molecular Devices, Sunnyvale, CA).

Immunostaining of neuronal cultures. Collagen VI (20 μg/ml), collagen I (20 μg/ml), or vehicle (4 mM borate buffer) was added to primary neuronal cultures from wildtype mice 1 h before exposure to oligomeric Aβ42 (45 μg/ml). After a 1 h exposure to Aβ42, cells were rinsed with PBS for 5 min, fixed with 4% PFA for 30 min at 25°C, washed, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were then incubated with a monoclonal anti-Aβ antibody (6E10, 1:1000, Signet, Dedham, MA) or no primary antibody overnight at 4°C, and incubated with Texas Red- or fluorescein isothiocyanate-conjugated antibodies (1:300, Vector Laboratories, Burlingame, CA) or Alexa-Fluor secondary antibodies (1:300, wavelength 594 or 488 nm, Invitrogen) for 1 h at 25°C. To double label cells for Aβ and collagen VI, a polyclonal rabbit anti-collagen VI antibody (1:1000, Abcam, Cambridge, MA) was added to the primary antibody incubation. To double label cells for Aβ and microtubule-associated protein 2 (MAP2, a neuronal marker), cells were sequentially immunolabeled with 6E10 and then anti-MAP2 (polyclonal rabbit 1:100, Chemicon, Temecula, CA). Cells were washed, stained with DAPI, and visualized by fluorescence or confocal microscopy. Low levels of cell-associated Aβ42 in collagen VI-treated neurons could not be captured in some images. No Aβ immunoreactivity was detected in cultures treated with Veh or collagen VI only or in Aβ-treated cultures without primary antibody (data not shown).

***In situ* hybridization.** After fixation at 4°C for 48 h, hemibrains were rinsed with PBS, placed in 30% sucrose in PBS at 4°C for 24 h, and coronally sectioned (30 μm) with a sliding microtome. Sections were stored at –20°C in cryoprotectant until use. All solutions were prepared with autoclaved water containing 0.1% diethyl pyrocarbonate and filtered (pore size: 0.22 μm). Antisense and sense cRNA probes corresponding to nucleotides 1330-2528 of Col6a1 cDNA (GenBank accession number Z18271) were generated from the linearized mCol6a1-4 plasmid[16] with T7 and T3 polymerase (Promega, Madison, WI) and premixed RNA-labeling nucleotides containing digoxigenin (Roche Molecular Biochemicals, Palo Alto, CA). The yield and integrity of cRNA riboprobes was confirmed by gel electrophoresis.

In situ hybridization was performed on floating sections as described[3]. After removal of cryoprotectant medium with PBS, floating sections were further postfixed in 4% phosphate-buffered paraformaldehyde, treated with 0.005% proteinase K in Tris-HCl (pH 8.0), EDTA, and 0.5% Tween for 15 min, and incubated with 1.335% triethanolamine, 0.175% HCl, and 0.25% acetic anhydride for 10 min. Between steps, sections were washed three times with PBS and 0.5% Tween at room temperature. Sections were then incubated with prehybridization buffer

containing 50% formamide, 5x SSC, 5x Denhardt's solution, salmon sperm DNA, and yeast tRNA for 4-6 h at room temperature. Riboprobes were diluted in the prehybridization buffer, heated to 70°C, and added to the sections. Hybridization was performed at 67°C for 16 h. Hybridized sections were washed once with 5x SSC and six times with 0.2x SSC at 67°C for 4 h. Sections were transferred to a buffer containing Tris, saline, and 0.5% Tween, blocked with 10% heat-inactivated sheep serum, and incubated overnight with sheep anti-digoxigenin-alkaline phosphatase (1:5000; Roche Molecular Biochemicals). The signal was visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (1:50; Roche Molecular Biochemicals) in NTMT buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Triton X-100). No signal was detected when sense *Col6a1* riboprobe was used (**Supplemental Fig. 3a**).

Quantitative Reverse Transcription (RT)-PCR. Total RNA was isolated from brain tissues or primary neuronal cultures with an RNA shredder and RNeasy Mini kits (Qiagen, Valencia, CA). After treatment with RNase-free DNase (Ambion) for 30 min at 37°C, total RNA was reverse transcribed with random hexamers and oligo(dT) primers. The expression level of Col6a1 relative to GAPDH or NSE was determined by SYBR green dye chemistry and an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA), as recommended by the manufacturer. The quality of primers and amplification reactions was verified by analysis of dissociation curves, slopes of standard curves, and reactions without RT. Human *Col6a1* expression was normalized to *NSE*, as used in human AD tissue to account for neuronal numbers or function[21]. Many neuronal markers decline in AD, including NSE (**Supplemental Fig. 1e)**. The following primers were used: mouse *Col6a1* (forward, 5'-TGCCCTGTGGATCTATTCTTCG-3'; reverse, 5'-CTGTCTCTCAGGTTGTCAATG-3'), mouse *Col6a2* (forward, 5'-CATCTCACCCCAGGAGCAGGAA-3'; reverse, 5'-TACACGTTGACTGGGCAGTCGG-3'), mouse *Col6a3* (forward, 5'-AACCCTCCACATACTGCTAATTC-3'; reverse, 5'-TCGTTGTCACTGGCTTCATT-3'), mouse *GAPDH* (forward, 5'-GGGAAGCCCATCACCATCTT-3'; reverse, 5'-GCCTTCTCCATGGTGGTGAA-3'), human *Col6a1* (forward, 5'-CGTCGATGCCATGGACTTTA-3'; reverse, 5'CGGTAGAAGCGGGTCACATAG-3'), human *Col6a2* (forward, 5'-CAACTGCCCAGAGAAGACCG-3'; reverse, 5'-GTGACGCTCTCCGAGGTGTC-3'), human *Col6a3* (forward, 5'-GTCAGACAACTGGACATGAGCC-3'; reverse, 5'-CGCGTGCTGCACAACTG-3'), human *NSE* (forward, 5'-CCATTTGACCAGGATGATTGG-3'; reverse, 5'-ACCCACAATCTGGATCCCTACA-3').

Western blot analysis. A McIlwain tissue chopper was used to cut hemibrains into 450-μm-thick horizontal sections from which the dentate gyrus was microdissected on ice. For protein quantitations, dentate gyrus samples from each hemibrain were pooled and homogenized on ice in buffer containing 320 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10 mM EGTA, 1% deoxycholate, 1 mM PMSF, Phosphatase Inhibitor Cocktails I and II (Sigma), and protease inhibitor mixture (Roche). Samples were briefly sonicated on ice and centrifuged at 5000 x *g* for 10 min. Equal amounts of protein (determined by Bradford assay) were resolved by SDS-PAGE on 4-12% gradient gels and transferred to nitrocellulose membranes. The following antibodies against collagen VI were used: AB7822 (Chemicon, Temecula, CA) and a custom-designed, rabbit polyclonal antibody against human and mouse α1(VI) (Invitrogen, Carlsbad, CA) that does not cross-react with α2(VI) or α3(VI) (**Supplemental Fig. 3 b,c**). Bands were visualized by enhanced chemiluminescence detection and quantitated densitometrically with Quantity One 4.0 software (Bio-Rad, Hercules, CA). For analysis of primary cultures, 20 μl of conditioned medium was analyzed per culture well as described above, and signal intensities were normalized to the total protein content of the respective cell pellets.

***Ex situ* atomic force microscopy (AFM).** HFIP-treated synthetic Aβ1-42 peptides (R-Peptide, Bogart, GA) were prepared as described[5, 14]. The stock solution of Aβ oligomers was diluted in PBS (pH 7.4) to a final concentration of 100 μg/ml. At time 0 h, a 5 μl sample was deposited on

freshly cleaved mica (SPI, West Chester, PA) and allowed to sit for 30 sec. The mica substrate with Aβ was washed with 200 μl of ultrapure water, and the sample was dried under a gentle stream of air. To measure Aβ at 24 h, the original Aβ preparation was agitated at 1000 rpm at 37^{o}C and sampled again as described above. For collagen VI samples prepared at pH 7-8 (in borate buffer) or at pH 2-3 (in acetic acid buffer), 10 μl of 20 μg/ml solutions were deposited on freshly cleaved mica, allowed to sit for 1 min, washed with 200 μl of water, and dried under a stream of air.

Aβ and collagen VI samples were imaged with a MFP3D scanning probe microscope (Asylum Research, Santa Barbara, CA). All images were taken with a silicon cantilever (Veeco, Santa Barbara, CA) with a nominal spring constant of 40 N/m and resonance frequency of ~ 300 kHz. Typical imaging parameters were: drive amplitude 150–500 kHz with set points of 0.7–0.8 V, scan frequencies of 2–4 Hz, image resolution 512 by 512 points, and scan size of 5 μm.

***In situ* AFM.** The MFP3D scanning probe microscope was equipped with a fluid cell filled with 180 μl of PBS buffer (pH 7.4). For experiments on collagen VI-coated substrates, collagen VI was deposited on mica as described above. Background images of bare or collagen VI-coated mica were obtained. Then, 20 μl of a monomeric preparation of Aβ^{22} was injected directly into the AFM fluid cell, resulting in a final Aβ concentration of 45 μg/ml, and the substrate surface was imaged continuously for 3 h. Images were obtained with a V-shaped oxide-sharpened silicon nitride cantilever (Veeco, Santa Barbara, CA) with a nominal spring constant of 0.5 N/m. Scan rates were set at 1-2 Hz with cantilever drive frequencies of ~8-12 kHz. Image resolution was 1024 by 1024 points. Aggregates of Aβ42 were quantified from four images in each condition at 145 min. AFM image analysis was performed with MatLab and its image processing toolbox (Mathworks, Natick, MA).

Statistical analyses. Statistical analyses were performed with SPSS 14.0 (SPSS, Chicago, IL). Differences between means were assessed with *t* tests. For nonparametic data (AFM analysis) intergroup differences were assessed with Fisher's exact test. Differences among multiple means were assessed by ANOVA and Tukey-Kramer *post hoc* test. Error bars represent standard error of the mean.

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